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PRESENTACIONES EN SIMPOSIOS

Cyanobacterial diversity and ecology on historic monuments in Latin America

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ABSTRACT. Cyanobacterial biofilms are complex communities of microorganisms that cause damaging activity on historic monuments. A combined molecular approach shows that cyanobacteria belonging to the order *Pleurocapsales* are the main colonizers at the Mayan site of Uxmal, Mexico, confirming previous microscopic and culture-based reports. An important, previously unrecognized non-cyanobacterial community comprising *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* has also been found in Uxmal. Cyanobacterial communities in Palenque were composed of over 10 species, mainly coccoid forms. A novel PCR method designed to directly amplify DNA from uncultured cyanobacterial cells on historic buildings in Brazil indicated that the identified cyanobacteria sequenced corresponded to their appropriate morphological groups (as defined by both the bacterial and botanical codes). However, their homologies with deposited sequences were, in general, low. Terrestrial cyanobacteria from stone surfaces in Brazil, again mainly coccoid, formed a distinct population that differed from the better-studied aquatic members. Overall, results here show demonstrate that coccoid cyanobacteria are the main colonizers on Latin American monuments under tropical and subtropical conditions and the assessment of their potential deteriogenic activity requires the further development of rapid molecular techniques. Polyphasic studies are essential to increase our knowledge of the diversity of terrestrial biofilms and of global microbial diversity.

Key words: Biodeterioration, biofilms, cyanobacteria, epiliths, historic buildings, microbial diversity.

RESUMEN. Las biopelículas cianobacterianas son comunidades complejas que ejercen una actividad deteriogénica importante en monumentos históricos. Un enfoque molecular combinado mostró que las cianobacterias del orden *Pleurocapsales* son los principales colonizadores de los monumentos Mayas en Uxmal México, confirmando reportes previos basados en estudios microscópicos y de cultivo. Una importante comunidad no cianobacteriana que comprende organismos de las divisiones *Proteobacteria*, *Firmicutes*, *Actinobacteria* y *Bacteriodetes* ha sido detectada también en Uxmal. Las comunidades cianobacterianas en Palenque por su parte, están compuestas de más de 10 especies, de las cuales los morfotipos cocoidales dominan. Un nuevo método basado en el uso de PCR aplicado directamente para amplificar ADN de células cianobacterianas no cultivadas obtenidas de monumentos en Brasil, indica que las cianobacterias secuenciadas corresponden con sus respectivos grupos morfológicos (tal como se define en los códigos bacterianos y botánicos). Sin embargo, sus homologías con secuencias depositadas fueron en general bajas. Las cianobacterias terrestres provenientes de superficies pétreas, nuevamente de tipo cocoide, forman una población distinta de sus contrapartes acuáticas mejor estudiadas. En general, estos estudios muestran que las cianobacterias cocoidales son los principales colonizadores en los monumentos históricos Latinoamericanos sujetos a climas tropicales y subtropicales y que la evaluación de su potencial actividad deteriogénica requiere del desarrollo de técnicas moleculares rápidas. Los estudios polifásicos son esenciales para incrementar nuestro conocimiento sobre la diversidad de biopelículas microbianas terrestres y de la diversidad microbiana global.

Palabras clave: Biodeterioro, biopelículas, cianobacterias, monumentos históricos, diversidad microbiana

INTRODUCTION

The influence of microorganisms in the deterioration of stone monuments has been frequently underestimated. The microbiota on building stones represents a complex ecosystem, which develops in various ways, depending on environmental conditions and the physicochemical properties of the material in question.[2,7,11,21,23]

A diverse community of microorganisms has been found on limestone and other types of stone, ranging from bacteria (including cyanobacteria), fungi, and algae to protozoa. Cyanobacteria are photolithoautotrophic organisms that can survive repeated cycles of drying and rehydration, and are able to withstand high UV levels.[24] These physiological attributes make them particularly important on exposed surfaces and they are considered the first colonizers in the succession of microbial populations on stone, although under certain circumstances oligotrophic heterotrophic microbes (bacteria and fungi) can develop without the need for nutrients from excreted metabolites or cyanobacterial biomass.[1,7] Phototrophic microorganisms may grow on the stone surface (epilithic phototrophs) or may penetrate some millimetres into the rock pore system (endolithic phototrophs).[15] These epilithic and endolithic organisms can potentially con-

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tribute to the breakdown of rock crystalline structures such as limestone, dolomite, sandstone and granite, among others[3,4,7,20,22] through the release of their metabolic products, such as inorganic and organic acids. In addition, extracellular polymeric materials (EPS), principally polysaccharides, act as glues, trapping dirt and other particulate materials, increasing the damaging effects of the biofilm. Given the hygroscopic nature of these biomolecules, they may cause mechanical stresses to the mineral structure due to shrinking and swelling cycles of these colloidal biogenic slimes inside the pore system, leading to the alteration of pore size and distribution, together with changes in moisture circulation patterns and temperature response.[7] The exacerbation of abiotic deterioration processes such as saline crystallization is also expected to occur.

Biopelículas microbianas asociadas a monumentos mayas en México y su papel en el deterioro pétreo

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Previous studies characterized the epilithic biofilms associated with Mayan monuments at Uxmal (Yucatan, Mexico) by microscopy and cultivation methods (Fig. 1). By using these methodological approaches, Ortega-Morales and co-workers[22] found that cyanobacterial populations of the genera *Xenococcus, Gloecapsa, Gloethece, Synechocystis* and *Synechococcus* were the dominating organisms in these biofilms. In agreement to our findings, Garcia-Miguel *et al.*,[9] found that cyanobacteria were the most abundant organisms associated with the Pyramid of the Great Jaguar at Tikal, Guatemala. These authors identified *Phormidium, Plectonema, Scytonema, Chlorogloepsis* and *Gloecapsa* as the most representative cyanobacterial genera. They also noted that, except for *Chlorella*, eucaryotic algae were absent from most samples.

On the other hand, Hoffmann,[17] in his review of algae in terrestrial habitats, reported that cyanobacteria capable of boring into limestone are of the genera *Gloeocapsa, Stigonema, Chroococcus, Aphanocapsa* and *Schizothrix.* We detected *Gloeocapsa* and *Synechocystis* (which includes members previously known as *Aphanocapsa*) in large numbers in our samples.

In our sites, coccoid forms were preponderant among the algae and the cyanobacteria. These findings were confirmed by microscopic analyses, where coccoid cells were seen in most of the analyzed samples (Fig. 2). These coccoid cells were embedded in a polymeric matrix, heavily covering the surfaces. In some locations the cells were covered with calcareous deposits, suggesting that migration of calcium from neighboring sites had occurred. Phototrophs deposit $CaCO_3$ in the day light and solubilize it in darkness due to a change in bicarbonate concentrations. The release of organic metabolites by cyanobacterial cell may be a major factor for calcium solubilisation for further deposition as calcium carbonate on cyanobacterial cells.

It is well known that most microorganisms from natural habitats are non-culturable. Therefore, previous studies based on cultivation-dependent methods may have failed to detect prevalent microorganisms. Therefore, a goal of this study was to gain a better understanding of the diversity of biofilm communities growing in different microenvironments on Mayan monuments by using cultivation-independent methods. Two community-profiling techniques, independent of each other, were employed on seven representative biofilm samples collected from three different buildings at the archaeological site Uxmal (Yucatan, Mexico): phospholipid fatty acid (PLFA) and PCR-single-strand-conformation polymorphism (SSCP) of partial genes encoding for RNA of the small subunit of ribosomes (SSU rRNA.[25] PLFA analysis is a community-level methodological approach currently used to determine the community composition and biomass.[21] However, for microbial community analysis, nucleic acid based methods that use SSU rRNA genes have a better resolution than PLFA. The nucleotide sequences of SSU rRNA genes can be linked directly to the phylogeny of an organism and allow a comparison of a retrieved sequence to previously found sequences available in databases.[16] The combination of PLFA and SSCP methods are a powerful tool to gain insight on the diversity of these epilithic biofilm communities under different expositional conditions.

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Table 1. Phototrophs identified in biofilms from interior walls in Mayan buildings in Uxmal.

Coccoid cyanobacteria	Coccoid algae	Filamentous cyanobacteria	Filamentous algae
Gloeocapsa	*Asterococcus*	*Chlorogloeopsis*	*Chrysocapsa*
Gloeothece	*Chlorella*	*Lyngbya*	*Heterococcus*
Myxosarcina	*Chlorococcum*	*Nodularia*	*Protoderma*
Pleurocapsa-group	Coccomyxa	*Nostoc*	*Stichococcus*
Synechococcus	*Dimorphococcus*		Trentepohliales
Synechocystis-like	*Planktosphaeria*		
*Xenococcus**	Unidentified Chlorococcales		

* *Cyanobacteria dominating the biomass of biofilms.*

Figure 1. *Uxmal is an important preclassical Mayan archaeological site founded in 800 BC. Left: Nunnery quadrangle, center: Magicien pyramid, right: Governor´s house.*

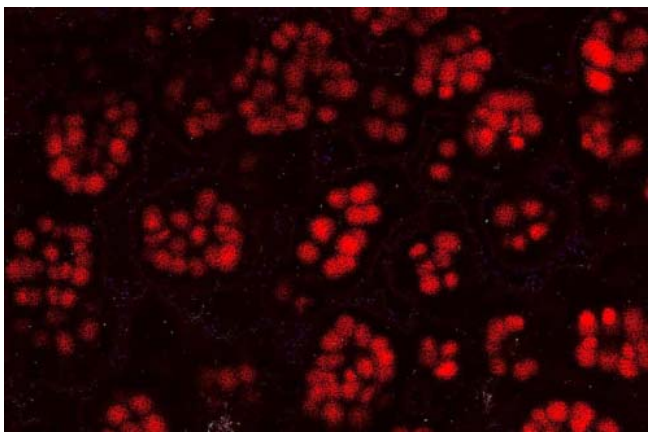

Figure 2. *Confocal laser electron microscopy image of colonial coccoidal cells, probably cyanobacteria. These cells were seen by scanning electronic microscopy (SEM) covered with calcareous deposits, suggesting the precipitation of solubilized calcium induced by phototrophic activity.*

Four distinct biofilm communities were identified on the basis of PLFA profiles analyzed by correspondence analysis. Biofilms 1, 3 and 7 were grouped in individual clusters while Biofilms 2, 4 5 and 6 were grouped in one specific cluster (Fig. 3). The PLFA profiles of the latter cluster of biofilm types (2, 4, 5 and 6) were dominated by C16:0 and C18:0 fatty acids along with the monounsaturates C18:1ω9, C18:1ω7c and C16:1ω7c, profiles similar to the cellular fatty acid composition reported for unicellular cyanobacteria from subsection II *Pleurocapsales*.[5] Interestingly, most of our cyanobacterial sequences detected by the SSCP approach were affiliated to the *Halothece* complex, which contains some representatives of the *Pleurocapsales*.[10]

A total of 35 sequences were retrieved in the course of this work (Table 1). Interestingly, the similarity of our partial rRNA gene sequences to public databases

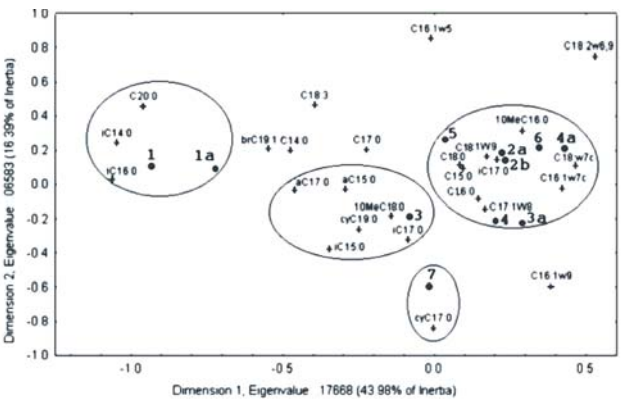

Figure 3. *Principal component analysis of PLFA from extant biofilms from Uxmal.*

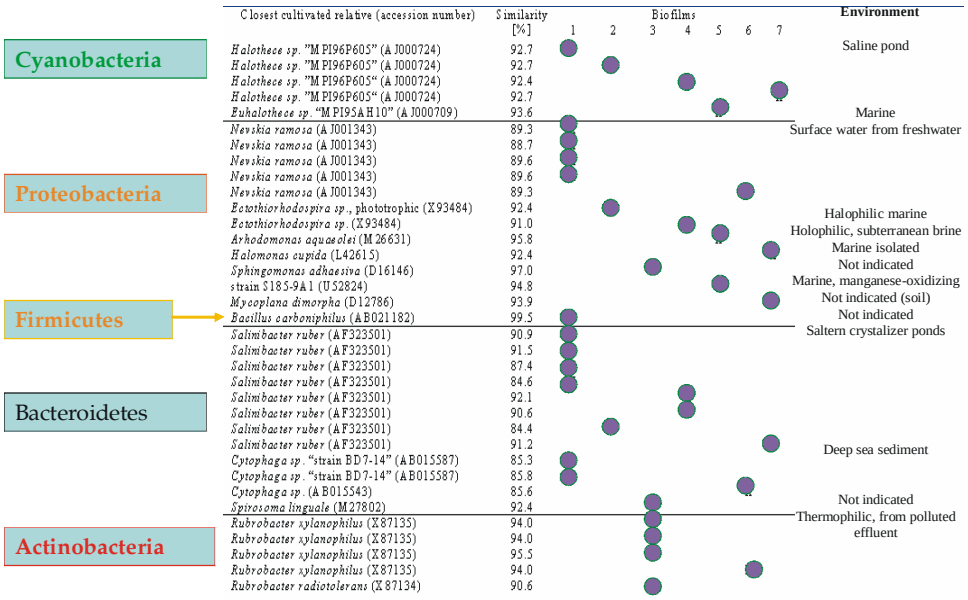

Figure 4. *Secuences retrieved from biofilms at Uxmal buildings.*

was relatively low (< 94 %). Five sequences (14.3 %) were related to the phylum *Cyanobacteria,* 12 sequences to *Proteobacteria* and *Bacteroidetes* (34.2 % each group) and 5 to *Actinobacteria* (14.3 %). Only one sequence (2.8 %) from the phylum *Firmicutes* was retrieved.

A total of 35 sequences were detected, which fell within 5 bacterial phyla (*Cyanobacteria, Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes*), from which most of the sequences (68 %) belonged to *Proteobacteria* and *Bacteroidetes*. In general, similar bacterial groups (*Cyanobacteria* and *Proteobacteria*) and certain specific genera (*Halothece)* colonized most of the surfaces analyzed (Fig. 4). However, specific organisms (*Nevskia ramosa* and *Salinibacter ruber* relatives) appear to be particularly associated with internal environments, characterized by low light and high water availability, while dryer, more illuminated walls were exclusively colonized by desiccation-tolerant *Rubrobacter*-related bacteria. Interestingly, most of the detected sequences were related to halophilic bacteria, suggesting that substratum salinity may have selected for this type of metabolism on these Mayan monuments. The low level of similarity of our sequences with public databases, suggests that many bacterial species remain to be discovered. Our results suggest that water availability, light regime and substratum salinity are important factors, which in part determine boundaries for biofilm formation and community composition in these habitats.

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Algae and cyanobacterial diversity and distribution patterns on mayan buildings in Palenque, Chiapas

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Part of the biodeterioration suffered in rural archeological areas is due to the growth of microorganisms such as bacteria, algae and fungi, which later favor the growth of higher organisms. Besides esthetic deterioration, algae form a film that makes maintenance and conservation of historical monuments very difficult.

For a better understanding of the role played by algae and cyanobacteria in the process of biodeterioration, they were studied in the Palace of Palenque (Chiapas) archeological zone over a period of a year. Because of the constitution of the buildings (calcareous material) and the prevailing climatic conditions (mildly humid, with a relative humidity between 42 and 84%), algal and cyanobacterial growth is diverse and abundant and is formed by subaerial species resistant to drastic changes in the environment, such as long periods of drought, high temperatures and prolonged exposure to the sun. Five basic forms of growth were found (mucilaginous, crusty, uneven, smooth and powdery), each composed of different groups of species of Chlorophyta and several genera of Cyanobacteria (Table 1).

The most abundant and widely distributed species found within the study area were *Scytonema guyanense*, *Asterocapsa* sp. and *Trentepohlia aurea*. In previous studies from this area,[26] 34 species were identified. Culture and microscopy of the uneven growth throughout the year shows a diverse morphology, associated with physiological changes (variation in external mucilage, the color, size and general morphology of the cells, etc.), which reflects

Table 1. Main cyanobacterial species detected at the Mayan site of Palenque.

Sample Area	Growth Appearance	Type of Growth	Dominant Species
Pillars (medallions) (O)	Bright green stains -pardo	Mucilaginous	*Gloeocapsa quaternata* *Gloeocapsa calcicola*
Medallions (O)	Bright green stains -olive	Crusty	*Gloeocapsa calcicola*
Corridor behind medallions (O)	Black growth	Uneven	*Scytonema guyanense*
Wall behind medallions (O)	Black and orange growth	Felt	*Scytonema guyanense* *Trentepohlia aurea*
Corridor at the back of medallions (S)	Black growth/underneath, bright green stains	Uneven/Mucilaginous	*Scytonema guyanense* *Chroococcus sp.*
Top of the Corridor roof (S)	Olive green stains	Soft	*Scytonema guyanense*
Basement (S)	Black and orange growth	Powdery/uneven	*Scytonema guyanense* *Choococcus sp*
Underground hallways (S)	Bright green growth/black	Mucilaginous	*Cyanothece sp* 2
Underground stairs	Bright green growth/orange	Mucilaginous	*Trentepohlia aurea* *Leptoyngbya sp.*
Underground (window E)	Bright grass green growth	Smooth	*Schizothrix sp*
Pillars (E)	Black growth	uneven	*Scytonema guyanense* *Gloeocapsa queternata* *Gloeocapsa calcicola*
Corridor dripping (E)	Black growth with carbonate deposits	Rough	*Gloeocapsa queternata* *Leptolyngbya sp*
Corridor basement (E)	Black and orange growth	Uneven, rough	*Scytonema guyanense* *Trentepohlia aurea*
Basement (N)	Black growth	Uneven	*Scytonema guyanense*
Corridor (between N/E face)	Black growth	Dusty	*Asterocapsa sp*
Tower hallway	Orange growth	Smooth mucilaginous	*Trentepohlia aurea*

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the different numbers and types of species, including many previously registered species.

The species distribution shows a pattern related to general illumination conditions (protected areas, or species resistant to high levels of irradiation), as well as with the general conditions of the substrate (species related to persistent humidity conditions or resistant to daily desiccation). The most relevant organisms are *Scytonema guyanense* and *Trentepohlia aurea*, species mostly found on exteriors, whilst most of the growths formed by unicellular organisms are found in shaded, relatively more humid areas. During the rainy season, the exterior growths change their specific composition for unicellular species, but without visibly modifying the general appearance of the growth. Shaded and permanently humid areas exist, covered with monospecific growths (*Asterocapsa* sp., *Cyanothece* sp. 2, *Gloeocapsa* sp.), but these species can also appear in other growths in lower proportions or in conditions protected from high irradiation. The evaluation of the algal development stages and the associated types of perennial structures also showed a different pattern for each species. Two types exist: those that develop structures during the rainy season (or in persistently humid conditions) and those that develop the structures during the dry season (in conditions with little humidity).

All of the elements previously mentioned can be considered for the design of growth control strategies for algae and cyanobacteria and consequently the conservation of these monuments.

Detección polifásica de cianobacterias en biopelículas de monumentos históricos en Brasil

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Samples were taken from the external surfaces of buildings in Porto Alegre and Ouro Preto, Brazil, using the non-destructive adhesive tape sampling method of Gaylarde and Gaylarde.[14] They were analyzed microbiologically by direct microscopic observation of rehydrated biofilms, culture on a basic mineral medium for phototrophs, and DNA sequencing after PCR using specific 16S rDNA cyanobacterial primers.[13]

It was found that the major biomass on the surfaces of these historic buildings, shown by direct observation of rehydrated biofilms, was almost always composed of coccoid cyanobacteria of Subsections I and II (formerly known as the *Chroococcales* and *Pleurocapsales*). These groups are poorly represented in the DNA databases and hence the polyvalent approach taken here was essential to determine their dominance. Even when a match was found between morphological and sequence data, the distances between the organisms on the dendrogram were large, showing that there was considerable evolutionary divergence between them.[6] This occurred, for example, with an organism found on a church in Ouro Preto (ccg46), identified as the genus *Chroococcidiopsis*. The organism, picked directly from a rehydrated biofilm as a separate colony and placed in an Eppendorf tube for direct PCR,[14] produced a good DNA partial sequence, which gave a 94% match with that of *Chroococcidiopsis* sp. BB79.2. (Fig. 1) shows the relevant part of the dendrogram and indicates that the divergence between these two Subsection II cyanobacteria is deeply rooted. The same can be seen for the closely-grouped members of the genus *Scytonema* detected in Porto Alegre (ccg 16, 25 and 26). Although they show a relationship with the DNA of *Scytonema hofmanni*, deposited in the data bank used by the BLAST facility, their distance from this species is large.

The results suggest that terrestrial organisms on historic buildings diverged from the aquatic ones some time ago, as has been previously suggested.[12] Their genome is considerably different, as might be expected in view of their very different distribution in the environment and their need to withstand desiccation and frequent high levels of UV light.

This analysis highlights the problems of attempting to use only morphological features for phylogenic analysis of cyanobacteria, since organisms with very close 16S rDNA sequences may have very different appearances, and vice-versa. Almost all of the cyanobacteria show changes in morphology in response to environmental conditions, and in the case of the heterocystous cyanobacteria and the baeocyte-forming groups, this morphological variation is always large. This means that detailed identification without culture under very standard conditions is not possible. The need for culture makes detection of major biomass in

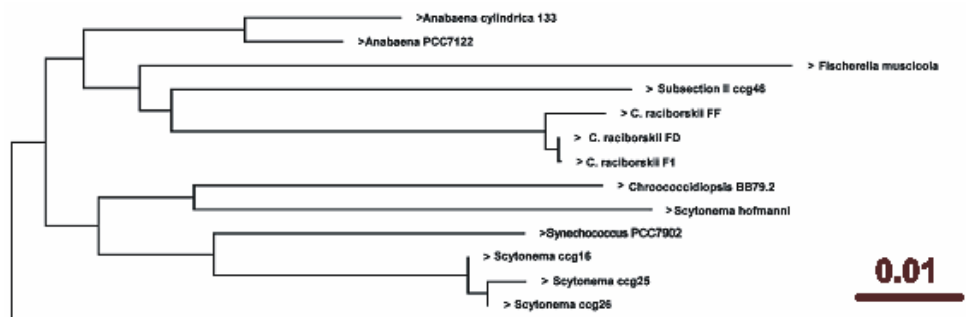

Figure 1. *Partial dendrogram showing retionship between (a) ccg46 (from an Ouro preto church) and* Chroococcidiopsis *BB79.2 (b) 3 Scytonema species from Porto Alegre (ccg 16, 25 and 26) and* Scytonema hofmanni *deposited DNA sequence.*

situ impossible, in many cases. Polyphasic methods, using a variety of techniques, are essential for the study of terrestrial biofilms.

The epilithic environment must be considered as a very distinct ecological niche. Terrestrial cyanobacteria from historic stone monuments surfaces, at least in Brazil, form a distinct population that differs from the better-studied aquatic members of this group. It is important that many more sequences for organisms from this important environment be deposited in the public data banks.

CONCLUSIONS

These studies show that coccoid cyanobacteria are the main colonizers on Latin American monuments under tropical and subtropical conditions. Water availability and light regime are the most important factors determining boundaries for biofilm formation, but salinity may influence community composition. These phototrophic biofilms on Mayan buildings may cause biodegradation by excretion of organic matter, supporting the growth of heterotrophic acid-producing bacteria and fungi and by active "boring" behaviour. Polyphasic studies are essential to increase our knowledge of the diversity of these biofilms and to assess the effect and efficiency of different treatments (i.e biocides) to control deteriogenic biofilm communities in cultural art work, which is valuable for conservation and restoration practices.

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